Brief Review

Id Family of Transcription Factors and Vascular Lesion Formation

Scott Forrest, Coleen McNamara

Abstract—Vascular smooth muscle cell (VSMC) modulation to a de-differentiated phenotype and proliferation are key components of vascular lesion formation. Understanding how these processes are regulated is essential to understanding the progression of vascular diseases such as atherosclerosis and in-stent restenosis. The Id family of helix-loop-helix (HLH) transcription factors has emerged as important regulators of cellular growth and differentiation. Recent published findings have implicated the Id proteins as important regulators of growth and phenotypic modulation in VSMC and in the vascular response to injury. In this review, we summarize what is known regarding how the Id proteins function to control cellular growth and differentiation and their role in vascular lesion formation. (Arterioscler Thromb Vasc Biol. 2004;24:1-7.)

Key Words:

Understanding VSMC biology is important for understanding the vasculoproliferative disorders (such as in-stent restenosis, vein graft failure, and transplant arteriopathy) and atherosclerosis. In the vasculoproliferative disorders, vessel wall injury results in the release of cytokines and mitogens, such as IL-1, thrombin, PDGF, FGF, Ang II, and transforming growth factor-β from platelets, inflammatory cells, and cells within the vessel wall.1–5 These potent factors can induce normally quiescent VSMC in the vessel wall to modulate to a less differentiated phenotype, re-enter the cell cycle, proliferate, and secrete extracellular matrix, contributing to neointimal formation.6

In atherosclerosis, lipid deposition with subsequent macrophage infiltration initiates a similar, although less accelerated, cascade of events. Accumulation of phenotypically modulated VSMC within fatty streaks over time leads to progression to fibrous plaques that may become occlusive to the vascular lumen.7,8 However, matrix secretion by VSMC late in atherosclerotic plaque development appears to increase plaque stability and thus protect against rupture and thrombosis—the cause of most acute coronary syndromes—so that VSMC may have both deleterious and protective roles in atherosclerosis.7,9,10 Therefore, a further understanding of the mechanisms regulating the phenotypic modulation and growth of VSMC may provide important insights that could lead to the development of strategies to limit the VSMC accumulation leading to neointimal formation and enhance VSMC proliferation and matrix production to stabilize advanced atherosclerotic plaques.

Regulated gene expression is essential for the maintenance of normal vessel structure and function and, not surprisingly, the expression of multiple genes is altered in the vasculoproliferative disorders and atherosclerosis. Recent comprehensive reviews broadly summarize regulation of gene transcription in the vessel wall and in VSMC and suggest that transcriptional modulation of genes involved in cholesterol and fatty acid metabolism, inflammation, and cell cycle progression may hold promise as favorable therapeutic approaches to limit atherosclerosis and the vascular response to injury.11,12 Transcription factors implicated in regulating these processes (such as the sterol responsive element-binding protein [SREBP], nuclear factor kappa B, E2F, and others) and their target genes (low-density lipoprotein receptor, adhesion molecules, cytokines, and cell cycle proteins) are known.11 However, the transcription factors and mechanisms that link phenotypic modulation and growth regulation in VSMC are poorly understood. Several classes of transcription factors are known or likely to be involved in the regulation of VSMC differentiation and, in some instances, proliferation.13 Notably, the HLH class of transcription factors is able to modulate both the expression of VSMC-specific and cell cycle regulatory genes. Subsequent studies provide evidence implicating the Id (inhibitor of DNA binding) class of HLH factors as regulators of growth and differentiation in VSMC and during vascular lesion formation.14,15 Thus, the focus of this review is on summarizing what is known regarding the role of the HLH proteins, particularly the Id factors, in regulating these processes in VSMC and during lesion formation. First, however, we briefly overview transcription factor classes and HLH factor involvement in the vasculature.
HLH Transcription Factors

Transcription factors can be broadly categorized into 4 superclasses—those containing basic domains ( superclass 1), those containing zinc-coordinated DNA binding domains ( superclass 2), those containing helix-turn-helix domains ( superclass 3), and β-scaffold factors ( superclass 4). In the vasculature, transcription factors from all 4 superclasses are expressed and contribute to vessel homeostasis. Transcription factors in superclass 1 can be further divided based on what specific motif(s) are present. One example is a basic HLH motif. Transcription factors containing the basic HLH (bHLH) motif all share a highly conserved HLH region, which mediates protein–protein interactions, as well as a basic DNA binding domain. bHLH factors activate transcription by forming homodimers or heterodimers that bind CANNTG consensus sequences, termed E-boxes, in the promoter region of responsive genes.

There are >240 HLH proteins identified; thus, a classification scheme based on tissue distribution, DNA-binding specificities, and dimerization capabilities has been devised. Class I HLH factors (known as E proteins) are bHLH factors that are expressed in many tissues including VSMC. E proteins such as E12, E47, HEB, and E2–2 can form homodimers or heterodimers with class II bHLH factors. Heterodimerization with class II bHLH proteins leads to activation of cell type-specific genes and cellular differentiation. Class II HLH proteins are tissue-restricted factors including MyoD and NeuroD. Factors belonging to this class (such as eHAND, dHAND, and capsulin/epicardin) have been implicated in the regulation of VSMC differentiation, although their specific target genes in VSMC are unknown. Class III HLH factors have a leucine zipper adjacent to the HLH motif (bHLH-LZ) and include c-Myc, E2F, and SREBP. Antisense-mediated c-Myc inhibition is capable of inhibiting VSMC growth and neointimal formation in response to injury in a rabbit and porcine model. E2F inhibition using “decoy” double-stranded oligonucleotides that contain consensus E2F binding sites reduced VSMC proliferation and lesion formation in response to balloon injury in porcine and rat arteries, as well as in response to vein grafting in rabbits and humans.

Id Proteins

Id proteins consist of 4 members (Id1–4) that belong to the class V HLH family of transcription factors. Unlike bHLH factors, the Id (Inhibitor of DNA binding) proteins lack the basic DNA binding domain altogether. Id proteins form inactive heterodimers with ubiquitously expressed class I bHLH factors and thus act as dominant-negative inhibitors of bHLH function (Figure 1). Whereas class I HLH factors are the primary target of Id proteins, Id also regulates class II HLH factor function because (with few exceptions) class II factors are incapable of forming homodimers and preferentially heterodimerize with class I E proteins. Class I and class II HLH factors regulate the transcription of cell cycle genes. Class I and class II factors also regulate the expression of cell type-specific genes and the differentiated phenotype. Thus, the Id proteins are likely candidates to mediate the cross-talk between pathways that regulate growth and pathways that regulate the differentiated phenotype in VSMC.

In addition to the common HLH domain that mediates Id dimerization, each Id protein has unique features that may regulate specific functional roles. Id2, Id3, and Id4, but not Id1, contain a consensus cdk2 phosphorylation site in their N-terminus and are phosphorylated by cyclin-ckd2 complexes, an event that alters the specificity of Id2 and Id3 in blocking bHLH dimers from binding E-box sequences in vitro. Phosphorylation of Id2 is required for transcriptional inhibition of p21 and cell cycle entry in VSMC. Furthermore, the C-terminal regions of the Id proteins differ dramatically (Figure 2). This is especially important because alterations in the C-termini of Id proteins have been shown to regulate protein stability and dominant-negative function. Two Id members, Id1 and Id3, have alternatively spliced isoforms. In rats these alternative isoforms, termed Id1.25 and Id3a, are found in cardiac and VSMCs. Interestingly, Id1.25 and Id3a appear to be generated by intron retention, resulting in alternative message products encoding unique C-termini. Id proteins can also dimerize with the class III bHLH-LZ protein SREBP and...
transcription factors that do not contain an HLH domain such as the ETS-domain factors, 51 MIDAI, 52 PAX-domain factors, 53 and pRb.54,55 The specific role of domains within the Id proteins and their role in mediating Id partner specificity are poorly understood.

**Ids and the Cell Cycle**

Id expression is upregulated after mitogen stimulation in a variety of cell types, and ectopic Id expression can promote cellular proliferation.38,55,56 However, whereas the Ids can all promote growth, and the general dominant-negative paradigm of Id function is common among family members, accumulating evidence indicates that the specific roles of these proteins in regulating growth are not redundant.55,56 Antisense inhibition of any 1 of the 4 Ids is sufficient to abrogate serum-stimulated proliferation in fibroblasts.56

Regulation of cell cycle progression represents a major means whereby the Id proteins affect cellular growth, and the specific mechanisms whereby this is accomplished are beginning to be elucidated. One major mechanism involves the regulation of cyclin-dependent kinase (cdk) inhibitors, including p16INK4a, p21Cip1, and p27Kip1. The cdk inhibitors modulate the formation and/or function of cyclin–cdk complexes that regulate progression of the cell cycle.57 Expression of p21Cip1 and p27Kip1 is elevated in terminally differentiated cells, and ectopic expression of p16INK4a, p21Cip1, and p27Kip1 results in cell-cycle arrest and a decrease of cellular growth in a variety of cell types, including VSMC.42,58,59 The promoter regions of p16INK4a and p21Cip1 contain multiple E-boxes, and several bHLH factors, including E47, are capable of stimulating p16INK4a and p21Cip1 transcription.57–59 Consistent with this paradigm, Id1, Id2, and Id3 can inhibit p21Cip1 and p16INK4a transcription in fibroblasts and VSMC.15,37,55 Therefore, by acting as dominant-negative transcriptional regulators of cyclin-dependent kinase inhibitors, the Ids can promote entry into the cell cycle and stimulate cellular growth.

A second mechanism whereby Id proteins promote cellular growth appears to be specific to Id2 and involves inactivation of the retinoblastoma protein (pRb). E2F, a transcription factor that activates a variety of genes required for cell cycle progression, is normally inactive by virtue of its association with pRb. Phosphorylation of pRb by cyclin/cdk complexes causes dissociation of the pRb/E2F complex, releasing E2F and allowing for cell cycle progression.57 Id2 (but not Id1 or Id3) is also able to promote complex dissociation, leading to enhanced cell cycle progression, via an interaction between the HLH region of Id2 and the pocket domain of pRb.54,55

**Ids Mediate Mitogen-Induced VSMC Growth**

Accumulating evidence suggests that the Id proteins are mediators of several mitogenetic factors involved in VSMC growth. Such factors act generally through receptor tyrosine kinases to stimulate classical mitogenic pathways, eg, the mitogen-activated protein kinase (MAPK) pathway.60 Receptor tyrosine kinase stimulation by PDGF or serum increases both Id expression and VSMC growth.56,61 Further, activation of protein kinase C or Ras is capable of upregulating Id3 expression, whereas this effect is blocked by either Ras or MAPK inhibition.62

Receptor tyrosine kinase-mediated MAPK activation results in increased transcription of early growth response gene product 1 (Egr-1). Egr-1 is upregulated in vivo in restenotic and atherosclerotic lesions, and Egr-1 inhibition reduces formation of both types of lesions in vivo.63–67 Interestingly, Egr-1 enhances expression of Id3 message, providing a link between mitogen or injury-induced Egr-1 and Id expression.68 Another immediate–early response gene, c-Myc, activates Id2 transcription. c-Myc is also upregulated after vascular injury, with c-Myc inhibition attenuating VSMC proliferation in vitro and restenosis in rat and porcine models of angio-plasty.24 c-Myc has recently been shown to bind the Id2 promoter and upregulate Id2 message levels.70 Of importance, Id2 is required for the mitogenic effects of c-Myc, because cells from Id2−/− mice, but not wild-type controls, are unresponsive to c-Myc stimulation.69 Angiotensin II (Ang II) is another VSMC mitogen. Ang II acts through AT1 angiotensin receptors to induce reactive oxygen species (ROS) production and growth in the VSMC.70 ROS act as signaling molecules, mediating the mitogenic effects of Ang II via activation of mitogenic pathways, including the MAPK cascade. Ang II is also capable of stimulating receptor tyrosine phosphorylation and activation of the MAPK pathway.61 Ang II is released after vascular injury, promotes VSMC growth and proliferation, and the mitogenic properties of Ang II have been shown to contribute to atherosclerosis.71–73 Mueller et al have recently identified Id3 as a message that is upregulated, in a MAPK-dependent manner, in cultured VSMC treated with Ang II.74,75 VSMCs treated with Ang II or ROS also displayed increased cell number and cell-cycle entry, and these effects were abrogated by Id3 antisense inhibition. Further, Ang II/ROS treatment decreased expression of p21Cip1 4 hours after treatment, coincident with Id3 induction.74 Thus, regulation of Id3 expression appears to be an important, indeed necessary, mechanism by which Ang II stimulates VSMC growth.

**The Ids and VSMC Differentiation**

In addition to their growth-promoting properties, the Id proteins can also act as negative regulators of cellular differentiation. Ectopic expression of Id proteins inhibits cellular differentiation in several cell line models, including myoblasts, epithelial cells, pre-adipocytes, and osteosarcoma cells, and expression of Id genes is significantly reduced on
Id proteins likely regulate differentiation via functional antagonism of bHLH factors well-characterized as promoters of cellular differentiation, such as MyoD and NeuroD.81

Modulation of VSMC phenotype, notably alteration of the VSMC differentiated state, is a hallmark of vascular lesion formation in both the vasculoproliferative disorders and the development of atherosclerosis.82 The primary function of differentiated VSMC is to contract; thus, expression of SM-specific contractile proteins, such as SM α-actin, is commonly used as an indicator of VSMC differentiation. Strong evidence for HLH involvement in the regulation of VSMC differentiation includes data published by Kumar et al, demonstrating that consensus bHLH DNA binding sites (E-boxes) are essential for SM α-actin promoter activation in vivo.14 Although several bHLH factors, including E12, E47, dHAND, and HES-2, are in fact expressed in VSMC, it is unclear whether these are involved in regulating VSMC differentiation in vivo.21,83–85 In vitro, ectopic expression of bHLH factors can drive SM α-actin expression in VSMC; importantly, coexpression of Id2 or Id3 abrogates this effect.14 It is therefore intriguing to think that the de-differentiation of VSMC after injury is mediated by the Id proteins. In vivo expression of VSMC differentiation markers, including SM α-actin, decreases after vascular injury at time points coincident with upregulation of Id2 and Id3.82 Therefore, the Id proteins are likely important mediators of the differentiated state of VSMC during lesion formation, although this has not yet been directly addressed.

Id Expression During Vascular Lesion Formation

Given that the Ids regulate VSMC growth and differentiation, it is not surprising that Id proteins have been implicated as modulators of vascular lesion development. Most of the in vivo data to date involve Id3. Id3 message expression in the vessel wall is significantly increased after balloon endothelial denudation in a rat carotid injury model. Id3, expressed at low levels in normal vessels, is increased within 3 days of injury and remains high through 14 days after balloon injury at the time points coincident with upregulation of Id2 and Id3.82 Therefore, the Id proteins are likely important mediators of the differentiated state of VSMC during lesion formation, although this has not yet been directly addressed.

In contrast to Id3, intron-containing Id3a message is absent from normal vessels and quiescent VSMC is strongly induced after balloon injury in rats and remains high through 28 days after injury. Interestingly, the human homologue of Id3a, Id3L, is expressed in advanced human atherosclerotic plaques, particularly in regions rich in VSMC (Figure 3).17 Moreover, gene delivery of Id3a, found to induce apoptosis and inhibit Id3 expression in VSMC, reduced the neointima-to-media ratio in response to balloon endothelial denudation in rats by 55%.15

Id2 expression is also modulated in vivo in a rat carotid injury model. Normally absent from the vessel wall, Id2 protein expression is induced by 6 days after balloon injury and remains high through 14 days. Like Id3, Id2 expression corresponds to peak cellular growth in the developing lesion. Id2 expression is declining by 28 days postinjury and is not seen in fully developed lesions.87 Expression of Id1 and Id4 in the vasculature has not yet been examined.

Id Involvement in Angiogenesis

Interesting in vivo data regarding Id function in the vasculature have recently been published by Lyden et al. This group generated Id3+/− Id1−/− double-knockout mice for developmental studies, but the mice are nonviable. They die in utero at E12.5 because of vascular abnormalities, which lead to intracranial hemorrhage. Results in earlier embryos demonstrated that the Id1−Id3 double-knockout mice have premature withdrawal of neuroblasts from the cell cycle and expression of neural-specific differentiation markers. However, Id1+/− Id3−/− mice were viable. An unexpected but exciting finding was that these mice had a marked attenuation of tumor vascularization and angiogenesis, providing the first in vivo evidence that the presence of Id3 is critical to the vasculature.88 These results are particularly relevant because angiogenesis is a process involving extensive VSMC migration, matrix secretion, and proliferation—themes held in common with the vascular response to injury—and is a critical process in atherosclerotic lesion progression.89–92

Summary

There is clear evidence that the Id proteins regulate VSMC growth and differentiation, key components of the vasculoproliferative disorders. Ids act to inhibit expression of genes that inhibit cell cycle progression, as well as genes specific for differentiated VSMC. Thus, Id proteins provide an important link between these 2 processes in the vasculature. Id expression is upregulated in developing restenotic lesions and is an important player in the signaling cascades activated by mitogens released after vascular injury.

The work reviewed provides evidence that Id proteins are key regulators of VSMC biology; however, important questions regarding the role of Ids in vascular disease remain unanswered. How are expression and function of the Id proteins coordinately regulated in VSMC in response to
injury? What are the other important cell cycle factors regulated by Id proteins? Do the Id proteins regulate other processes in VSMC such as migration or matrix production? What bHLH factors do Id proteins regulate in VSMC and in the vessel wall? Do Id deletions or mutations alter susceptibility to vascular disease? Clearly, much further research in this area is needed, because a more complete understanding of how Id proteins regulate VSMC growth and differentiation and vascular lesion formation is essential to elucidating the molecular mechanisms of lesion development.

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